AD_____

Award Number: DAMD17-02-P-1115

TITLE: Interagency Botulism Research Coordinating Committee

PRINCIPAL INVESTIGATOR: Eric A. Johnson

CONTRACTING ORGANIZATION: University of Wisconsin
Madison, Wisconsin 53706-1490

REPORT DATE: November 2002

TYPE OF REPORT: Final Proceedings

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	November 2002	Final Proceedings (22 Oct 02 - 25 Oct 02)

4. TITLE AND SUBTITLE
Interagency Botulism Research Coordinating Committee

5. FUNDING NUMBERS
DAMD17-02-P-1115

6. AUTHOR(S):
Eric A. Johnson

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Wisconsin
Madison, Wisconsin 53706-1490

E-mail: easjohnso@facstaff.wisc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

none provided

14. SUBJECT TERMS:
botulism, research

15. NUMBER OF PAGES
60

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Interagency Botulism Research Coordinating Committee (IBRCC)
October 22 – 25, 2002
Pyle Center, Madison, WI

Michael Adler
USAMRICD
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010
410-436-1913
410-436-8377
michael.adler@amedd.army.mil

Suman Adler
USAMRICD
3100 Ricketts Point Road
APG, MD 21010
410-436-1913
410-436-8377
suman_adler@hotmail.com

S. Ashraf Ahmed
USAMRIID
1425 Porter Street
Fort Detrick, MD 21702-5011
301-619-6299
301-619-4238
syed.ahmed@amedd.army.mil

Jaime Anderson
USAMRIID
1425 Porter Street
Fort Detrick, MD 21702-5011
301-619-6009
301-619-6911
jaime.anderson@amedd.army.mil

Joanne Andreadis
Centers for Disease Control & Prevention
1600 Clifton Rd, MS - G29
Atlanta, GA 30333
404-639-4331
404-639-4290
jsa9@cdc.gov

K. Roger Aoki
Allergan, Inc.
Neurotoxins
2525 Dupont Drive
Irvine, CA 92612
714-246-5413
714-246-4374
aoki_roger@allergan.com

Stephen Arnon
CA Dept Health Services
DCDC, Infant Botulism Treatment/Prevention
2151 Berkeley Way, Room 506
Berkeley, CA 94704-1011
510-540-2646
510-540-3205

Jason Barash
CA Dept Health Services
DCDC, Infant Botulism Treatment/Prevention
2151 Berkeley Way, Room 506
Berkeley, CA 94704-1011
510-540-2646
510-540-3205

Karen Crawford
List Biological Labs, Inc.
501-B Vandell Way
Campbell, CA 95008
408-866-6363
408-866-6364
crawford@listlabs.com

Bibhuti DasGupta
University of Wisconsin
Food Research Institute
1925 Willow Drive
Madison, WI 53706
608-263-6806
608-263-1114

Sean Dineen
University of Wisconsin
Food Microbiology & Toxicology
1925 Willow Drive
Madison, WI 53706-1187
608-263-9420
608-263-1114
ssdineen@wisc.edu

Lucia Fenicia
Instituto Superiore della Sanità
Viale Regina Elena
Roma, Italy 00161
39-06-4990-2319
39-06-49387101
fenicia@iss.it

Joseph Ferreira
Food and Drug Administration
60 8th St NE
Atlanta, GA 30309
404-253-2216
404-253-2216
jferreir@ora.fda.gov

Giovanna Franciosa
Istituto Superiore della Sanità
Viale Regina Elena 299
Roma, Italy 00161
39-06-4990-2810
39-06-49387101
francios@iss.it

Joseph Francis
Allergan, Inc.
2525 Dupont Drive
Irvine, CA 92612
714-246-4179
714-246-4374
francis_joe@allergan.com

Ingrid Friberg
CA Dept Health Services
DCDC, Infant Botulism Treatment/Prevention
2151 Berkeley Way, Room 506
Berkeley, CA 94704-1011
510-540-2646
510-540-3205

Sebastian Hielm
University of Helsinki
Food and Environmental Hygiene
P.O. Box 57
FIN-00014 Helsinki University
Helsinki, Finland FIN-00014
358-9-19149715
358-9-19149718
sebastian.hielm@helsinki.fi

Dianne Hodges
Allergan, Inc.
2525 DuPont Drive
Irvine, CA 92612
714-246-5170
714-246-5101
hodges_dianne@allergan.com

Eric Johnson
University of Wisconsin
Food Microbiology & Toxicology
1925 Willow Drive
Madison, WI 53706
608-263-7944
608-263-1114
eajohnso@wisc.edu

Jean Johnson
University of Wisconsin
Food Microbiology & Toxicology
1925 Willow Drive
Madison, WI 53706
608-263-4047
608-263-1114
jljohns2@wisc.edu

James Keller
Food and Drug Administration
Lab of Bacterial Toxins
29 Lincoln Dr.
HFM 437
Bethesda, MD 20892
301-496-4695
kellerj@cber.fda.gov

Meir Kende
US Army Medical
Research Institute of Infectious Diseases
1425 Porter Street
Fort Detrick
Frederick, MD 21702-5011
301-619-7494
311-619-2348
meir.kende@amedd.army.mil

Riikka Keto-Timonen
University of Helsinki
Food and Environmental Hygiene
P.O. Box 57
FIN-00014 Helsinki University
Helsinki, Finland
358-9-191-49698
358-9-191-49718
rikka.keto@helsinki.fi

Timo Klewitz
University of Hannover
Inst of Technical Chemistry
Callinstr. 3
Hannover, Germany D-30167
49-0-511-762-2381
49-0-511-762-3004
klewitz@iftc.uni-hannover.de

James McGee
Centers for Disease Control & Prevention
National Botulism Reference & Surveillance Lab
1600 Clifton Rd, MS - G29
NE Bldg 17, Room 1219
Atlanta, GA 30333
404-639-4331
404-639-4290
jfcgee@cdc.gov

Xintian Ming
Rhodia, Inc.
P.O. Box 592
Madison, WI 53701
608-224-3125
608-224-3130

Vicki Montgomery
USAMRIID
Division of Toxinology
1425 Porter St
Fort Detrick, MD 21702
301-619-4255
301-619-2348
vicki.montgomery@amedd.army.mil

Mari Nevas
University of Helsinki
Food and Environmental Hygiene
P.O. Box 57
FIN-00014 Helsinki University
Helsinki, Finland
358-9-191-49744
358-9-191-49718
mari.nevas@helsinki.fi

Pauline Nol
USGS/Natl Wildlife Health Ctr
6006 Schroeder Rd
Madison, WI 53711
608-270-2489
608-270-2418
pauline_nol@usgs.gov

Melvin Oka
Allergan, Inc.
2525 DuPont Drive
Irvine, CA 92612
714-246-5170
714-246-5101
oka_mel@allergan.com

Sean O'Malley
Hawaii Biotech, Inc.
Chemistry Group
99-193 Aiea Heights Dr., Suite 200
Aiea, HI 96701
808-486-5359
808-487-7341
somalley@hibiotech.com

Michael Peck
Institute of Food Research
Norwich Research Park, Colney
Norwich, UK NR47UA
44-1603-255000
44-1603-507723
mike.peck@bbsrc.ac.uk

Susan Smith
USGS/National Wildlife Health Center
6006 Schroeder Rd.
Madison, WI 53711
608-270-2453
608-270-2418
susan_r_smith@usgs.gov

Theresa Smith
USAMRIID
Toxinology And Aerobiology Div
1425 Porter Street
Fort Detrick, MD 21702
301-619-4229
301-619-2348
theresa.smith@det.amedd.army.mil

Jeremy Sobel
Centers for Disease Control & Prevention
National Center for Infectious Diseases
1600 Clifton Rd, MS - A38
Atlanta, GA 30333
404-639-2206
404-639-2205
jsobel@cdc.gov

Haim Solomon
US Food & Drug Admin
5100 Paint Branch Parkway
College Park, MD 20740
301-436-2013
301-436-2644
hsolomon@cfsan.fda.gov

Rebekah Starner
Battelle Memorial Institute
505 King Avenue, JM-3
Columbus, OH 43201-2693
614-424-5240
614-424-5469
starner@battelle.org

David Steele
Chem/Bio Medical Systems
1436 Porter Street
Fort Detrick, MD 21702
301-619-7128
301-619-7230
david.steele@det.amedd.army.mil

Ray Stevens
The Scripps Research Institute
10550 N. Torrey Pines Road, SR101
La Jolla, CA 92037
858-784-9483
858-784-9416
stevens@scripps.edu

Subramanyam Swaminathan
Brookhaven National Laboratory
Biology Department
Biology Department
Upton, NY 11973
631-344-3187
631-344-3407
swami@bnl.gov

Interagency Botulism Research Coordinating Committee Meeting

October 22–25, 2002

Hosted by: Eric Johnson
Food Research Institute, UW-Madison

Conference site: The Pyle Center,
702 Langdon Street, Madison, Wisconsin 53706

Overview of the Program

Tuesday, Oct. 22, 2002		Alumni Lounge, Pyle Center	
	Reception	5:00 – 7:00 p.m.	
Wednesday, October 23, 2002		Auditorium, Pyle Center	
	Epidemiology	9:00 a.m. – 12:00 p.m.	S. Maslanka, moderator
	Wildlife Botulism	1:00 – 2:30 p.m.	T. Rocke, mod.
	Assay Development and Prophylaxis	3:00 – 5:30 p.m.	J. Keller / I. Henderson, mod.
(dinner on your own Wednesday evening)			
Thursday, October 24, 2002		Auditorium, Pyle Center	
	Basic Science: Structure / Function	8:00 – 10:00 a.m.	F. Lebeda, moderator
	Basic Science: Genetics and Physiology	10:30 a.m. – 12:00 p.m.	E. Johnson, mod.
	Basic Science: Mode of Action	1:00 – 3:00 p.m.	M. Adler, mod.
	Food Safety	3:30 – 5:30 p.m.	M. Peck, mod.
	Poster Session / Reception	5:30 – 6:30 p.m.[Alumni Lounge]	
	Banquet	6:30 – 8:30 p.m. [Alumni Lounge]	Haim Solomon, speaker
Friday, October 25, 2002		Auditorium, Pyle Center	
	Clinical	8:00 – 9:30 a. m.	S. Arnon, moderator
	Hot Topics	10:00 – 11:00 a.m.	E. Johnson, mod.
	Wrap-up / Discussion	11:00 – 12:00 p.m.	E. Johnson, mod.
	Depart	noon	

Program: Wednesday, October 23, 2002 Auditorium, Pyle Center

Session	Title	Presenter	Affiliation
Epidemiology Susan Maslanka 9:00 a.m. – 12:00 p.m.	Botulism Surveillance Summary, USA 2001	Jeremy Sobel, Susan E. Maslanka	CDC, Atlanta, GA
	Foodborne Botulism in the United States: Old and New Vehicles Causing a Rare Disease, 1990–2000	Nicole A. Tucker	CDC, Atlanta, GA
	Foodborne Botulism on the Rise in the Republic of Georgia, 1980–2000	Nato Tarkhashvili	National Centers for Disease Control, Tbilisi, Republic of Georgia
	- - - - - - Break 10:30 – 11:00 a.m. - - - - - -		
	A Central California Coastal "Hot-Zone" of Infant Botulism	Ingrid K. Friberg	California Dept. Health Services, Berkeley, CA
	First Case of Infant Botulism in Finland, Misdiagnosed as Cot Death	Mari A. Nevas	Veterinary Medicine, University of Helsinki, Helsinki, Finland
- - - - - - Lunch 12:00 – 1:00 p.m. - - - - - -			
Wildlife Botulism Tonie Rocke 1:00 – 2:30 p.m.	Recent Outbreaks of Type E Botulism in Waterbirds on the Great Lakes	Tonie E. Rocke	USGS National Wildlife Health Center, Madison, WI
	The Use of PCR for Detection of Type C and E Toxin Genes in Wetland Sediments	Judy L. Williamson	USGS National Wildlife Health Center, Madison, WI
	The Epizootiology of Type C Botulism at the Salton Sea	Pauline Nol	USGS National Wildlife Health Center, Madison, WI
- - - - - - Break 2:30 – 3:00 p.m. - - - - - -			
Assay Development and Prophylaxis James Keller and Ian Henderson 3:00 – 5:30 p.m.	Utility of an in vitro ELISA in the Botulism Clinical Laboratory	Susan E. Maslanka	CDC, Atlanta, GA
	Recombinant Antibodies for Botulism Therapy	James D. Marks	Univ. California, San Francisco, CA
	Progress Towards a Recombinant Botulinum Multivalent Vaccine	Ian Henderson	DynPort Vaccine Co., Frederick, MD
	Uptake of Botulinum Neurotoxin into Cultured Neurons	James E. Keller	FDA, Bethesda, MD
	Sensitive Immunochromatographic Assay for Detection of Botulinum Toxin Type D	Timo Klewitz	Univ. Hannover, Hannover, Germany

[dinner on your own Wednesday evening]

Botulism Surveillance Summary, USA 2001

J. Sobel, N. Tucker, and S. Maslanka

Centers for Disease Control and Prevention
Atlanta, GA

The Centers for Disease Control and Prevention maintain surveillance for all syndromes of botulism. Sources of data include reports from: state health departments, the CDC botulism laboratory, the Infant Botulism Treatment and Prevention Program, CDC epidemiologists, and the CDC botulism antitoxin release database.

One-hundred and sixty-seven cases of botulism were reported to CDC in 2001. Thirty one cases of foodborne botulism were reported, of which 20 (65%) were caused by toxin type A, eight (26%) by toxin type E, two (7%) by toxin type B, and one by toxin type F. The median age of patients was 46 years. One death was reported. There were three multi-case outbreaks. One affected 16 persons in Texas and was caused by commercial chili that was time- and temperature-abused in a salvage food store; two, affecting four and two persons, respectively, in Alaska, were caused by Alaskan Native foods (beaver and whitefish).

One-hundred and twelve cases of infant botulism were reported. Toxin type B accounted for 67 (60%) cases and toxin type A for 45 (40%) cases. The median age of patients was 14 weeks; one death was reported.

Twenty-three cases of wound botulism were reported. Twenty-two cases were caused by toxin type A, and one case by toxin type B. The median age of patients was 41 years. All but one were reportedly drug injectors. One death was reported, in a patient who suffered a wound in a motor vehicle accident.

One case of adult colonization botulism was reported. The case was caused by toxin type F. The patient, a 45 year-old woman with a history of obesity and stomach surgery, survived.

Foodborne Botulism in the United States: Old and New Vehicles Causing a Rare Disease, 1990–2000

N. Tucker, J. Sobel, A. Sulka, and S. Maslanka

Centers for Disease Control and Prevention
Atlanta, GA

Background: Foodborne botulism is a potentially lethal neuroparalytic disease caused by ingestion of preformed toxin of *Clostridium botulinum*.

Methods: We reviewed CDC surveillance data and published and unpublished case and outbreak investigations from 1990–2000 to identify incidence, demographic and geographic trends, contaminated foods, and toxin types. We defined an episode of foodborne botulism as one or more cases linked to a single exposure.

Results: One-hundred and sixty episodes resulting in 263 cases of foodborne botulism were reported during 1990–2000. Fifty-eight (36%) episodes with 103 (39%) cases occurred in Alaska. Median age of all cases was 48 years and 154 (59%) cases occurred among females. The case fatality rate was 4%. The median number of cases per year was 23 (range: 17–43), the median number of episodes per year was 14 (range: 9–24), and the median number of cases per episode was 1 (range: 1–17). A marked seasonal peak occurred in Alaska during May–August. Toxin type A was identified in 51% of cases overall and caused 72% and 87% of cases east and west of the Mississippi River (excluding Alaska), respectively. Toxin type E caused 90% of cases in Alaska. A food was implicated in 126 (79%) episodes. In the continental United States, the implicated food in 70 (91%) episodes was non-commercial, most commonly home-canned vegetables (44%). Two episodes, affecting 8 and 17 persons respectively, were restaurant-associated. Five episodes affecting a total of 10 persons were caused by commercial foods: Moloha, an unevicerated salted fish; low-acid clam chowder, and a bean dip, both sold in low-oxygen containers that were left unrefrigerated by consumers; a commercially produced burrito; and store-purchased fresh surgeon fish cooked at home. All episodes in Alaska were caused by traditional Alaskan Native foods, including seal oil (29%) and fermented sea mammals (22%).

Conclusions: Home-canned vegetables and Alaskan Native dishes remain the leading causes of foodborne botulism in the United States. Restaurant-associated episodes cause a disproportionate number of cases. Existing prohibitions on the sale of unevicerated uncooked fish should be enforced, as should strict regulation of fresh-fish storage time and temperature. Non-sterile, low-acid, high water activity commercial foods packaged in airtight containers should have additional barriers such as acidity or salinity to inhibit *C. botulinum* germination.

Foodborne Botulism on the Rise in the Republic of Georgia, 1980–2000

G. Katsitadze, M. Moiscrafishvili, N. Chakvetadze, P. Imnadze,
E. Jhorjholiani, T. Zardiashvili, M. Chokheli, and N. Tarkhashvili

National Centers for Disease Control
Tbilisi, Republic of Georgia

Background: Botulism is a rare disease in the U.S., but is reported worldwide. The Republic of Georgia, an independent, ethnically diverse nation in Western Asia, has previously reported the highest rate of foodborne botulism in the world, but the epidemiology has never been formally described.

Methods: Georgia's National Centers for Disease Control (NCDC) conducts national surveillance for botulism. Prior to 1996, NCDC collected summary reports from regional epidemiology offices and only investigated large outbreaks. In 1996, NCDC began collecting detailed histories on all botulism cases ascertained by regional epidemiology offices. We analyzed data from summary reports and vital statistics for the period from 1980-2000, and combined this data with case histories and hospital records for the period from 1996-2000.

Results: From 1980-2000, NCDC ascertained 806 cases of botulism. From 1980-1995, a total of 563 botulism cases (mean 35.2 cases / year) and 36 deaths (mean 2.3 cases / year) were reported. From 1996-2000, the average number of cases and deaths increased; 243 cases (mean 48.6 cases / year) and 18 deaths (mean 3.6 cases / year) were reported. This corresponds to an annual incidence of 1.1 per 100,000 persons. There was marked regional variability with the vast majority of cases ascertained in the eastern and central regions of the country. Of the 243 persons with botulism from 1996-2000, 109 (45%) were female, and 224 (92%) were over 15 years of age. Most cases (107, 44%) occurred in the winter, and the least occurred in the spring (36, 14.8%). 210 cases (86%) occurred as part of outbreaks (>1 ill case). The largest of these outbreaks involved 23 persons in 1999. All reported cases of botulism were foodborne. Home conserved vegetables were implicated in 197 (81%) cases and smoked fish in 45 (18.5%). The most commonly implicated vegetables were tomatoes 44 (18.10%). Laboratory testing was performed for 121 (50%) cases; of these, 111 (92%) were positive for botulism. All laboratory-confirmed cases associated with smoked fish were type E, and all cases associated with vegetables were type B. Anti-toxin was administered to 238 (97.94%) patients; of the five that did not receive anti-toxin, four survived.

Conclusion: Foodborne botulism is a major public health problem in Georgia. NCDC has begun a multi-year project to improve physician and public education, public health surveillance, laboratory diagnostic capacity, and anti-toxin distribution. Reducing the burden of botulism will involve learning which food conservation practices increase the risk of botulism and will require developing a cheap, effective, culturally appropriate, home-based intervention.

A Central California Coastal "Hot Zone" of Infant Botulism

I. K. Friberg and S. S. Arnon

Infant Botulism Treatment & Prevention Program,
California Department of Health Services
Berkeley, CA
ifriberg@dhs.ca.gov

Infant botulism is a rare disease in the United States with approximately 100 laboratory-proven cases reported annually, resulting in a national incidence of 2.0 per 100,000 live-births. Several states, including California, have an annual incidence approximately 3-5 times the national average. The recent identification of a Mid-Atlantic "hot-zone," a rectangle of 50 miles x 150 miles encompassing parts of five states (NY, PA, NJ, DE, MD) which has an annual incidence of 8.5 per 100,000 live-births, prompted an analysis of infant botulism incidence and epidemiology in similarly circumscribed regions of California. In California infant botulism cases are identified through physician requests for diagnostic testing and, since 1992, for the investigational orphan drug BIG-IV, both of which are provided solely by the California Department of Health Services.

The first modern-era case of infant botulism was recognized in California in Monterey County in the spring of 1976, thereby initiating statewide epidemiologic surveillance of the disease. Over the past 26 years Monterey County and the two central coastal counties immediately to its south, San Luis Obispo and Santa Barbara, have been three of the four highest-incidence counties statewide (among those counties reporting at least three cases). The overall incidence of infant botulism in these three counties is $19.0/10^5$ live-births, approximately three times the California statewide incidence ($6.5/10^5$ live-births) and approximately 10 times the national incidence. These three counties form a rectangle approximately 200 miles long and 50 miles wide. We analyzed the descriptive epidemiology of all 77 infant botulism cases that occurred in this region from March 1976 through August 2002. Cases were distributed among the three counties in direct proportion to the number of births in each county. Cases occurred in all months and peaked between August and October. 77% were type A and 23% were type B, not significantly different from the statewide proportions. 48% were female and 52% male. 79% were Caucasian and 21% Hispanic, a distribution representative of the underlying population. The mean age of onset was 19.2 weeks, in contrast to the 14.0 weeks mean of all the remaining California cases ($p<0.0001$). 86% of the patients were still primarily breast-fed at onset, as compared to 60% of other California cases ($p<0.0001$). Honey ingestion prior to onset by "hot-zone" cases over the 26-year study period did not differ significantly from the rest of California (8% vs. 12%, respectively; $p=0.62$)

The recognition of infant botulism "hot-zones" on both the east and west coasts of the United States suggests that infant botulism cases are not equally likely to occur in all areas where *Clostridium botulinum* spores are found. Further investigation of regional environmental (e.g. soil types) and sociodemographic factors (e.g., breast-feeding rates) in these "hot-zones" may advance the understanding, and eventually the prevention, of infant botulism cases nationwide.

First Case of Infant Botulism in Finland, Misdiagnosed as Cot Death

Mari Nevas[1], Miia Lindström[1], A. Virtanen[2], Sebastian Hielm[1],
E. Vuori[2], and Hannu Korkeala[1]

[1]Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine,
Helsinki University, Helsinki, Finland

[2]Department of Forensic Medicine, Faculty of Medicine,
Helsinki University, Helsinki, Finland

Clostridium botulinum spores may cause an infectious toxigenic disease called infant botulism. When ingested by a child aged less than one year, spores may germinate, grow and produce toxin in the intestinal lumen. In the most fulminant form of infant botulism, death may occur with no previous signs, thus resembling a sudden infant death syndrome (SIDS). In January 2002 an unexpected death of a ten-week old boy was diagnosed as SIDS according to the autopsy findings. The child had been healthy despite of a two-day constipation period at the age of four weeks, but no signs of constipation preceded the death. Death occurred a few hours after the child had been fed, the final feeding being slower than normal. Serum and autopsy sections of the intestine and spleen were collected as specimens for detection of *C. botulinum*. The amount of feces available for toxin detection was insufficient. The tissue samples were cultured in TPGY broth and on EYA and BSM agar plates and the serum was used for toxin detection by a bioassay. *C. botulinum* type B was detected by PCR from the intestinal wall sample and the strain was isolated. Toxin production of the strain was confirmed. Other tissue samples were negative for *C. botulinum* and the serum did not contain botulinum toxin. Honey, which is considered a significant risk factor for infant botulism, was not included in the infant's diet. No spores of *C. botulinum* were found from the infant formula fed to the child, but type B spores were detected and also isolated from the dust collected from the vacuum cleaner of the household. The genetic similarity of the isolates from the vacuum cleaner and from the infant was confirmed by PFGE and RAPD typing methods.

Recent Outbreaks of Type E Botulism in Waterbirds on the Great Lakes

Tonie E. Rocke and Grace S. McLaughlin

USGS, National Wildlife Health Center
Madison, WI 53711

For the past 4 years, type E botulism has been confirmed in waterbirds from the Great Lakes during several noticeable die-off events. The birds involved in these events include gulls, loons, mergansers, and other ducks. Although type E botulism is considered the most likely etiology of the die-offs, other pathogens have been detected as well, including type C botulism and Salmonella. The largest events have occurred on Lake Erie, where it has been estimated that over 35,000 individuals have died in the last 4 years, but smaller numbers of birds have also been confirmed with type E botulism from Lake Huron. Large numbers (thousands) of dead mud puppies (salamanders) have been reported on Lake Erie as well numerous dead fish, including rock bass, smallmouth bass, carp, suckers, perch, gobies, catfish and sheepshead. Unfortunately, no cause of mortality has been determined for the mudpuppy and fish kills, largely because the poor condition of specimens has hampered diagnostic evaluations; however a number of hypotheses have been put forward including type E botulism, low oxygen, and algal toxins. The continued problems with type E botulism in Lake Erie may be related to the presence of nonnative zebra and quagga mussels. These die-offs will be discussed in relation to previous type E botulism outbreaks that occurred on the Great Lakes in the 1960's and 80's.

The Use of PCR for Detection of Type C and E Toxin Genes in Wetland Sediments

Judy L. Williamson and Tonie E. Rocke

USGS, National Wildlife Health Center
Madison, WI 53711

In 1999 a reconnaissance survey was conducted to determine the distribution of *Clostridium botulinum* type C in the Salton Sea ecosystem. Sediment samples were collected from various locations within and surrounding the Salton Sea and tested for the type C toxin gene using a semi-nested PCR assay. We found that vegetative cells of *C. botulinum* type C are widely distributed throughout the Salton Sea ecosystem, are not highly abundant, and demonstrate a seasonal trend in which the prevalence of the *C.botulinum* organism is highest in the spring. In this study, we also collected measurements for five different environmental parameters in the water column at the time of sediment collection. These parameters included 1) temperature, 2) conductivity, 3) dissolved oxygen, 4) pH, and 5) redox potential, all which have been previously associated with type C botulism outbreaks in waterfowl in freshwater wetlands. An analysis was conducted to determine the relationship between these parameters and the presence of the C_1 toxin gene in the sediments. Although seasonal trends or patterns were identified for most of the environmental factors, there was little evidence, with the exception of water pH, to support a correlation between any of the environmental variables and the presence of the type C botulism toxin gene. We are currently conducting a similar survey on the distribution of *C. botulinum* type E toxin gene in these sediments, and will be using a quantitative PCR assay, which was specifically developed for the C_1 toxin gene, to determine toxin gene levels.

The Epizootiology of Type C Botulism at the Salton Sea

Pauline Nol, Judy L. Williamson and Tonie E. Rocke

USGS, National Wildlife Health Center
Madison, WI 53711

In recent years the Salton Sea has been the site of massive mortality events involving pelicans and other fish-eating birds. During the summer of 1996, type C avian botulism killed nearly 20,000 birds, over half of which were western white pelicans (*Pelecanus erythrorhynchos*) and close to 1,200 being endangered California brown pelicans (*Pelecanus occidentalis californicus*). Smaller botulism die-offs have occurred every year thereafter. Three years ago, NWHC launched a comprehensive study to investigate the ecology of avian botulism at the Sea. In 1999, we conducted a yearlong survey to assess prevalence of *Clostridium botulinum* type C in the sediments around the Sea at different times of the year. In 1999, 2000 and 2001, we collected tilapia (*Oreochromis mossambicus*) at various sites during botulism outbreaks in order to determine the presence of toxin-producing bacteria in their intestines, as well as to look for the presence of type C botulinum toxin in their blood and intestines. We found that toxin-producing cells were present in the sediments during winter and spring and are detected mostly in agricultural drains and at the river deltas. This suggests that the sediments are not the primary source of toxin during botulism outbreaks, which occur during the summer. Within the tilapia population we noted differences among the years in regard to prevalence of the active bacteria. Tilapia captured in 2000 had a significantly higher prevalence of toxin-producing bacteria than tilapia in 2001. Prevalence in 2000 was notably, though not significantly higher than in 1999. These differences correspond to the increased severity of the 2000 outbreak in pelicans compared to 1999 and 2001. This information, in conjunction with spatial data obtained from affected pelicans, as well as population data from tilapia and pelicans, will provide insight into the dynamics of this unique disease system.

Utility of an in vitro ELISA in the Botulism Clinical Laboratory

Susan Maslanka[(1)], Joanne Andreadis [(1)], & Joseph Ferreira[(2)]

[(1)]Centers for Disease Control and Prevention, Atlanta, GA
[(2)] Food and Drug Administration, Southeast Regional Laboratory, Atlanta, GA

A previously studied amplified ELISA system for botulinum toxin detection was modified by substituting digoxigenin-labeled IgG and anti-digoxigenin horse-radish peroxidase conjugate for biotin-labeled IgG and streptavidin alkaline phosphatase. Botulinum toxins were captured on antitoxin coated microtiter plates then bound by type-specific digoxigenin-labeled goat antitoxin. Surface-bound digoxigenin residues were detected using anti-digoxigenin Fab fragments conjugated to polymerized horse-radish peroxidase and TMB substrate. This modification of the amplified ELISA reduced the number of steps of the procedure and allowed the analyst to monitor the progress of the absorbance without sacrificing sensitivity. The sensitivity of the test for the detection of purified neurotoxin was approximately 0.12 ng/ml for botulinum toxin types A, B, and F. The sensitivity of the type E test was 0.25 ng/ml. Endpoint dilutions of culture supernatants demonstrated that most cultures ≥ 10 MLD/ml of botulinum toxin could be detected. Since most *C. botulinum* strains produce ≥100 MLD/ml in broth media, this method may be useful for screening culture supernatants for botulinum toxin. This method is currently undergoing testing to determine its use in the clinical diagnostic laboratory.

Recombinant Antibodies for Botulism Therapy

I. Geren[1], R. Tsai[1], A. Razai[1], J. Lou[1], R. Blake[3], A. Nowakowski[1], D. Powers[1], T. Smith[2], L. A. Smith[2], and J. D. Marks[1]

[1]Department of Anesthesia, University of California, San Francisco
marksj@anesthesia.ucsf.edu
[2]Toxinology Division, USAMRIID, Ft. Detrick, MD
[3]Xavier Univeristy, LA

To generate therapeutic antibodies (Ab) capable of potently neutralizing Botulinum neurotoxin A (BoNT/A), single chain Fv (scFv) phage antibody libraries were constructed from mice immunized with the binding domain (HC) of BoNT/A and humans immunized with pentavalent BoNT toxoid. As reported at last years IBRCC, a total of 44 unique Abs were generated and characterized with respect to: 1) affinity; 2) epitope recognized; and 3) ability to neutralize BoNT/A in vitro. scFv binding to 3 non-overlapping epitopes were found to prolong the time to paralysis in vitro. Co-administration of two scFv led to more potent toxin neutralization. To determine the potency of toxin neutralization in vivo, IgG were constructed from the V-genes of the C25, S25, and 3D12 scFv. This yielded chimeric (mouse V domains-human C domains) Ab for C25 and S25 and a fully human Ab for 3D12. At a dose of 50 ug, C25, S25, or 3D12 caused a delay in time to death of mice receiving 20 or 100 LD50s of toxin. At best, only 10% of mice survived at either toxin dose. When administered as pairs of Abs at a total dose of 50 ug, animals survived challenge with between 100 and 2000 LD50s of toxin (S25+C25, S25+3D12, or C25+3D12). The combination of all three Abs was 10 times more potent than the most potent Ab pair and has a neutralizing capacity of 45 IU/mg of Ab.

To generate a human compatible therapeutic antibody, we searched a phage antibody library generated from mice transgenic for the human immunoglobulin locus for human neutralizing antibodies to replace S25 and C25. The antibody S25 could be replaced in the three Ab mixture with an antibody (B4) binding to a spatially neighboring epitope, with a 2 fold increase in potency. The antibody C25 was humanized by CDR grafting, since no replacement antibody could be found in the transgenic mouse library, with a further 2 fold increase in potency (all mice receiving 50 ug of 3D12 + B4 + humanized C25 challenged with 40,000 LD50s of Hall toxin survived). Data will be presented regarding: 1) the mechanism of Ab synergy in neutralizing toxin; and 2) the role of non-HC Ab in neutralizing toxin.

We conclude that BoNT can be potently neutralized with a combination of monoclonal antibodies and that this approach offers a route for immunotherapy and prophylaxis for Botulism.
This work was supported by: DAMD17-94-C-4034 and DAMD17-98-C-8030

Progress Towards a Recombinant Botulinum Multivalent Vaccine

Ian Henderson

DynPort Vaccine Company, LLC
Frederick, MD 21702

DynPort Vaccine Company, LLC (DVC) is a biopharmaceutical company located in Frederick, Maryland, and is the Prime Systems Contractor for the US Department of Defense Joint Vaccine Acquisition Program (DOD-JVAP). DVC has the responsibility for the advanced development and licensure of the processes for the manufacture of a range of vaccines with the US Food and Drug Administration (FDA) designed to protect against several biological agents. One of these is a recombinant multivalent vaccine designed to protect against the effects of intoxication by botulinum neurotoxins. The current vaccine is designed to protect against botulinum neurotoxin serotypes A and B. The vaccine will comprise of two antigens derived from the C-terminal domains of the heavy chains of the neurotoxins. The antigens are expressed in the methylotrophic yeast *Pichia pastoris* under the control of the methanol inducible promoter *AOS1*. Through collaboration with several industry partners, manufacturing processes for the production of Purified Drug Substance for each vaccine antigen have been optimized and demonstrated at the intended manufacturing scale. In parallel, studies have also been undertaken to derive a stable liquid formulation for this vaccine containing a suitable adjuvant. This work is currently focused on the filing of an Investigational New Drug Application with FDA, and initiation of phase 1 clinical trials for the bivalent vaccine candidate, in 2003. Progress towards this goal will be described.

Uptake of Botulinum Neurotoxin into Cultured Neurons

James E. Keller

Food and Drug Administration
Bethesda, MD

Botulinum neurotoxin (BoNT) serotypes A and E cleave separate sites near the C-terminus of the neuronal protein SNAP-25 impairing synaptic function and resulting in muscle paralysis. Despite a common substrate, BoNT-A remains functional in neurons for many months whereas BoNT-E acts for only days. In the present study, we determined that 20-fold more BoNT is taken up by cultures of dissociated spinal cord neurons during K+-induced stimulation than in the absence of stimulation, as determined by SNAP-25 Western blot analysis. After uptake into an endosomal compartment, the toxin translocates this membrane and enters the cytosol. The extent of toxin-induced damage is concentration-dependent but the rate of damage is independent of toxin concentration. Both serotypes cleaved similar amounts of SNAP-25 at concentrations between 1-4 mouse LD50 units per μL, which constitutes the first demonstration that dose-dependent substrate cleavage in neuronal cell culture correlates with in vivo lethality. Damage to SNAP-25 follows a delay of about 0.4 hr after toxin uptake. Inhibition of the vacuolar ATPase with bafilomycin A1 (bafA1) prevents cleavage of SNAP-25. Application of bafA1 at measured times after toxin exposure demonstrates that toxin translocation from the acidic compartment is complete about 1.5 hr following toxin uptake. BafA1 blocks BoNT-A and –E translocation with IC50 values of 28 ± 3 nM and 494 ± 52 nM, respectively, indicating that BoNT-A requires significantly more ATPase activity than BoNT-E for successful translocation. Furthermore, bafA1 acts with Hill coefficients of 2.3 ± 0.4 for BoNT-A and 1.1 ± 0.2 for BoNT-E, suggesting that BoNT-A requires two, and BoNT-E one proton for efficient translocation. These differing pH-related properties i.e., bafA1 IC50 & Hill coefficient, may account for the different paralytic effects of type-A and -E observed in vivo.

Sensitive Immunochromatographic Assay for Detection of Botulinum Toxin Type D

Timo Klewitz[1], H. Beer[2], F. Gessler[3],
T. Scheper[1], and K. Pflanz[2]

[1] Institut für Technische Chemie, Callinstr. 3, Universität Hannover, D-30167 Hannover
[2] Sartorius AG, Weender Landstr. 94-108, D-37075 Göttingen
[3] Institut für Tropentierhygiene, Kellnerweg 6, Universität Göttingen, D- 37077 Göttingen

A highly sensitive dipstick assay for botulinum toxin Type D detection is developed, based on a sandwich format using two primary antibodies of distinct specificities and one secondary antibody. One of the primary antibodies was conjugated with colloidal gold (detector reagent), the secondary antibody (capture reagent) was immobilized within a test line on a Unisart membrane. In combination with an effective sample pre-treatment the toxin formed a mobile sandwich complex with the two primary antibodies. When applying the pre-treated liquid sample onto the test strip the mobile sandwich complex moves with the sample towards the absorbent pad. Within the test line the mobile sandwich complex is immobilized and concentrated by the secondary antibody resulting in a distinct red test line. Using a silver enhancement method the sensitivity of the test signal could be further increased to 50 pg/ml, with a total assay time of less than 5 hours.

Program: Thursday, October 24, 2002 Auditorium, Pyle Center

Session	Title	Presenter	Affiliation
Basic Science: Structure / Function Frank Lebeda 8:00 – 10:00 a.m.	Role of Zinc in Botulinum Neurotoxin — Structural or Catalytic?	Subramanyam Swaminathan	Brookhaven National Laboratory, Upton, NY
	Structural View of Botulinum Neurotoxin in Numerous Functional States	Ray Stevens	Scripps Institute, La Jolla, CA
	Autocatalytic Fragmentation of Botulinum A Neurotoxin Light Chain Does Not Affect the Protein's Catalytic Activity	S. Ashraf Ahmed	USAMRIID, Fort Detrick, MD
	BotDB: A Database for Clostridial Neurotoxins	Frank J. Lebeda	USAMRIID, Frederick, MD
- - - - - - Break 10:0 – 10:30 a.m. - - - - - -			
Basic Science: Genetics and Physiology Eric Johnson 10:30 a.m. – 12:00 p.m.	Role of Incubation Atmosphere on Toxin Production in Clostridium Type C and D Strains	Frank Gessler	Institute Applied Biotechnology in the Tropics, Göttingen, Germany
	Genetics of Toxin Complexes in *Clostridium botulinum* Type A Strains	Sean Dineen	Food Research Institute, University of Wisconsin, Madison, WI
	Relative Quantification of the Type B Neurotoxin Gene Expression in *Clostridium botulinum* Using Real-Time RT-PCR	Maria K. Lövenklev	Lund Institute of Technology, Lund University, Lund, Sweden
- - - - - - Lunch 12:00 – 1:00 p.m. - - - - - -			
Basic Science: Mode of Action Michael Adler 1:00 – 3:00 p.m.	Botulinomics — Botulinum Neurotoxins Do Not Cause But Flaccid Muscular Paralysis	H. Böhnel and F. Gessler	Institute for Tropical Animal Health, Georg-August University, Göttingen, Germany
	Hn-33 Enhances the Endopeptidase Activity of Botulinum Neurotoxin A and E Against Brain Synaptosomal SNAP-25	Shashi K. Sharma	US FDA, CFSAN, College Park, MD
	Persistence of Botulinum Neurotoxin Serotypes A and E in Rat Extensor Digitorum Longus Muscle	Michael Adler	USAMRIID, Aberdeen Proving Ground, MD
	Molecular Steps in Botulinum Action — Known Knowns and Known Unknowns	Bal Ram Singh	Univ. Massachusetts, Dartmouth, MA
- - - - - - Break 3:00 – 3:30 p.m. - - - - - -			
Food Safety Michael Peck 3:30 – 5:30 p.m.	Inhibition of Toxin Production of Nonproteolytic *Clostridium botulinum* Type B in Cooked Sausages by Nitrite	Riikka O. Keto-Timonen	Veterinary Medicine, Univ. Helsinki, Helsinki, Finland
	Formulating Low-Acid Foods for Botulinal Safety	Ann E. Larson	Dept. Food Microbiology and Toxicology, Univ. Wisconsin, Madison, WI
	Thermal Inactivation of Nonproteolytic *Clostridium botulinum* Type E in Fish	Miia Lindström	Food and Environmental Hygiene, Univ. Helsinki, Helsinki, Finland
	An Exposure Assessment of the Foodborne Botulism Hazard in Gnocchi, a Minimally Processed Refrigerated Potato Product of Italian Origin	Michael W. Peck	Institute of Food Research, Norwich, England
Poster Session / Reception 5:30 – 6:30 p.m.	[see separate listing of presenters]		Alumni Lounge
Banquet 6:30 – 8:30 p.m., Alumni Lounge	**Haim Solomon, speaker**		

Role of Zinc in Botulinum Neurotoxins — Structural or Catalytic?

S. Swaminathan, S. Eswaramoorthy and D. Kumaran

Brookhaven National Laboratory
Upton, NY 11973
swami@bnl.gov

Clostridium botulinum produces seven distinct serotypes (A – G) of botulinum neurotoxins (BoNTs) which cause botulism leading to flaccid paralysis and eventual death. Botulinum neurotoxins bind to the presynaptic membranes of neuronal cells *via* gangliosides and a second protein receptor, are internalized and then are translocated into the cytosol by a pH dependent mechanism where they attack and cleave one of the three proteins of SNARE complex. This inhibits the formation of SNARE complex which is required for vesicle docking and fusion and hence blocks neurotransmitter release causing flaccid paralysis. Though they cleave specific substrates at specific peptide bonds, they all contain a zinc-binding motif (HExxH) and are classified as zinc endopeptidase. The role of zinc has been investigated by biochemical methods and the presumed structural changes caused by the removal of zinc have been analyzed by spectroscopic methods but no x-ray structure is available on zinc-depleted toxin, so far. Apo toxin crystals were prepared either by titrating the pH in the crystal or by treatment with EDTA before crystallization. Results from these two studies will be presented.

Research supported by the Chemical and Biological Non-proliferation program - NN20 of the U.S. Department of Energy and the U.S. Army Medical Research Acquisition Activity (Award No. DAMD17-02-2-0011) under prime contract No. DE-AC02-98CHI0886 with Brookhaven National Laboratory.

Structural View of Botulinum Neurotoxin in Numerous Functional States

Raymond C. Stevens

Department of Chemistry and Molecular Biology
The Scripps Research Institute
La Jolla, CA 92037

We have determined the 3-dimensional structures of the bacterial released botulinum neurotoxin complex (therapeutic form), the pure 150-kDA Botulinum neurotoxin, the 50 kDa catalytic domain (light chain) responsible for cleaving synaptic vesicle proteins, and the 50 kDa catalytic domain bound to the synaptic vesicle protein target. By determining the structure in these different functional states, we are able to reconstruct the journey the toxin undertakes when it is released from the bacterium all the way until it cleaves its target in neuronal cells. For example: (1) We are able to understand which parts of the toxin are accessible when in the complex form. (2) A comparison of the holotoxin catalytic domain and the separated catalytic domain structure shows a rearrangement of three active site loops. This rearrangement exposes the catalytic domain active site to cleave its target. (3) The synaptic vesicle protein bound to the toxin structure illustrates two distinct binding regions and explains the high specificity of each botulinum neurotoxin for its synaptic vesicle protein. This observation also provides an explanation for the proposed cooperativity between full-length substrate binding and catalysis and demonstrates for the first time the mechanism of synaptobrevin proteolysis, which prevents neurotransmitter release. (4) Finally, we are able to understand how certain inhibitors stop the toxin activity, while others do not work.

Autocatalytic Fragmentation of Botulinum A Neurotoxin Light Chain Does Not Affect the Protein's Catalytic Activity

S. Ashraf Ahmed and Leonard A. Smith

Department of Immunology and Molecular Biology
Toxinology and Aerobiology Division, USAMRIID
Fort Detrick, MD 21702
Syed.ahmed@amedd.army.mil

Highly purified recombinant zinc-endopeptidase light chain of botulinum neurotoxin serotype A underwent proteolytic processing at the C-terminus and proteolytic fragmentation at the middle of the molecule during storage (J Protein Chem 20: 221-231 (2001). An SDS-stable dimer of the light chain accumulated before the fragmentation reaction. Zinc enhanced the rate of proteolysis and the metal chelator, TPEN, or a specific competitive inhibitor peptide, CRATKML, drastically reduced the proteolysis. These results suggested that C-terminal processing and fragmentation of the light chain are enzymatic and autocatalytic. We have mapped the sites of proteolysis and found that the light chain is an endopeptidase with broad specificity.

In the presence of zinc, when most (>95%) of the light chain had undergone autocatalytic fragmentation, the preparation retained 35% of it's original catalytic activity against a SNAP-25-derived synthetic peptide. A >95% fragmented light chain obtained in the absence of added zinc after 13 days of incubation at room temperature retained 100% of its original activity. On the other hand, in the presence of glycerol, the light chain did not display autocatalysis but retained 100% of the original activity. These results suggested that the active-site environment was not affected significantly by autocatalytic fragmentation of the light chain. Circular dichroism and tryptophan fluorescence spectra of the fragmented light chain were compared to those of the intact light chain to find secondary and tertiary structural differences. The optimum pH (4.5) for autocatalysis was significantly lower than the optimum pH (7.3) for catalysis, suggesting a low-pH-indiced, rate-limiting conformational change of the light chain. An indication of such a conformational change is reflected in the increased (8-12 °C) thermal stability at low pH in the absence of $ZnCl_2$ or at high pH in the presence of $ZnCl_2$.

BotDB: A Database for Clostridial Neurotoxins

Frank J. Lebeda

USAMRIID
Frederick, MD 21702

A clostridial neurotoxin database is being constructed that contains structural and biochemical data for these toxins, their substrates, and inhibitors. Its intent is to provide a focal point for new and experienced investigators to obtain information from a variety of diverse resources. The object-oriented schema of this database was modified from aCHEdb, which, in turn, was derived from ACEDB, a genomic database originally developed for *C. elegans*. The present α-test version (BotDB) for PC WINDOWS platforms contains selected amino acid sequences, results from x-ray diffraction studies of neurotoxin crystal structures, and circular dichroic studies of these neurotoxins in aqueous solution. Non-structural information includes steady-state ligand binding and inhibition constants, detection assay, and other biochemistry data. Relevant literature references are hyperlinked to the corresponding data via the WWW. Information retrieval is accomplished by several built-in query utilities including a novel query language. BotDB will be available on CD to interested research groups.

Role of Incubation Atmosphere on Toxin Production in Clostridium Type C and D Strains

Frank Gessler[1] and Helge Böhnel[2]

[1] Institute for Applied Biotechnology in the Tropics at the Georg-August-University
Kellnerweg 6, 37077 Göttingen, Germany
e-mail fgessle@gwdg.de

[2] Institute for Tropical Animal Health, Georg-August-University
Kellnerweg 6, 37077 Göttingen, Germany
e-mail hboehne@gwdg.de

Clostridium botulinum belongs to the anaerobes. However there are quite a variety of gas compositions that can be used to incubate the bacteria. We have studied the effects of the incubation atmosphere on growth and especially on toxin production in various type C and D strains.

Cultures were subcultivated in the respective atmosphere for three times. The last passage was incubated over a period of ten days. Aliquots were regularly checked for toxicity in the culture supernatant. For toxicity testing a magnetic beads assay was used. The assay principle is based on a sandwich technique with a monoclonal catching and a polybiotinilated, polyclonal detection antibody. Streptavidin-HRP and Tetramethyl benzidine were used as conjugated and substrate.

From each tube bacteria were counted as well.

Genetics of Toxin Complexes in *Clostridium botulinum* Type A Strains

Sean S. Dineen, M. Bradshaw, and E. A. Johnson

Food Research Institute
University of Wisconsin–Madison
Madison, WI 53706

Botulinum neurotoxin (BoNT) exists as one of the components of a progenitor toxin complex. The genes encoding the components of the progenitor toxin complex are arranged in a cluster, which varies in composition and organization among the various serotypes and strains. Over the past decade, a significant amount of BoNT gene cluster sequence data has been obtained. The published complete DNA sequences of the entire BoNT gene clusters are only available for a limited number of strains of serotypes C and D, while the Sanger project is determining the entire genomic sequence of *Clostridium botulinum* Hall A (ATCC 3502). Very little sequence data is available for the regions flanking the BoNT gene clusters, although such analyses could reveal genes involved in regulation and mobilization of the BoNT genes. Kinetic studies in our laboratory of BoNT expression in three *C. botulinum* strains that produce BoNT/A (62A, Hall-*hyper*, and NCTC 2916) showed significant differences in synthesis of the BoNT cluster components. Since genes within and flanking the toxin clusters could also affect expression, we performed sequencing studies in these three strains. Comparisons of the sequences determined in this study provide insight into the organization, regulation, genetic transfer, and evolutionary history of genes for botulinum neurotoxin type A complexes.

Relative Quantification of the Type B Neurotoxin Gene Expression in *Clostridium botulinum* Using Real-Time RT-PCR

Maria Dahlenborg Lövenklev[1], E. Borch[2], E. Holst[3], and P. Rådström[1]

[1] Applied Microbiology, Lund Institute of Technology, Lund University, Lund, Sweden
[2] SIK, Ideon, Lund, Sweden
[3] Dept. of Medical Microbiology, Dermatology and Infection, Lund University, Lund, Sweden

Introduction: Clostridia capable of producing botulinum neurotoxin are widely distributed in nature and their presence in the food environment is known. Traditionally the risk of botulism is reduced either by heat killing of spores and/or bacteria cells or by preventing bacterial growth in the food. Preventative measures rely mainly on lowering a_W, a reduction in pH or maintaining food at a temperature below that *Clostridium botulinum* can grow. More knowledge about how different environmental signals, regulates the toxin gene expression will be necessary for an assessment of the botulinum hazard in different kinds of food i.e., REPFEDs (Refrigerated Processed Foods of Extended Durability).

In this study, a new method using real-time reverse transcription PCR for relative quantification of mRNA transcripts of the type B toxin gene expression is described. Furthermore, strains of *C. botulinum* were screened for their patterns of growth and toxin gene expression by using the developed quantitative real-time PCR. This method will be used to investigate how physiological factors regulate the toxin gene expression in *C. botulinum.*

Experimental procedures: Internal fluorogenic probes, based on the TaqMan™ principle, have been designed to study in real-time the amplification of cDNA using the LightCycler™ instrument. For relative quantification analysis, the type B toxin gene expression is normalised by a non-regulated reference gene expression. The reference gene used is part of the 16S rRNA gene. Six strains of *C. botulinum,* 3 proteolytic strains (ATCC 7949, ATCC 17841 and Atlanta 3025) and 3 non-proteolytic strains (Johannesson 105-66, Eklund 17B and Eklund 2B) were screened for their patterns of growth and toxin gene expression in a tryptone/peptone/yeast (TPY) medium with the addition of 4% glucose and 1% of each maltose, cellobiose and starch to promote the toxin production. Seventeen samples were collected for total RNA extraction during growth between 0 h and 50 h. Prior to reverse transcription with Superscript™ II Reverse Transcriptase all samples were treated with DNase to remove any DNA contamination. For cDNA synthesis 0.5 µg total RNA from each sample was used. The cDNA was then amplified with *Tth* DNA polymerase in the LightCycler™ and the pattern of relative expression determined for each of the six strains.

Results and discussion: In the proteolytic strains, an increase in expression was observed in the early stationary phase (between 5 h to 7 h of growth) and again in the death phase of the growth, (after 30 h to 36 h). As for the non-proteolytic strains there was also an increase in expression in the early stationary phase (after 5 h). Afterwards the level of expression decreased and continued to decline during the 50 h. Work is in progress to relate the toxin expression with the protein production (ELISA) and the formation of the active toxin (mouse bioassay).

Botulinomics — Botulinum Neurotoxins Do Not Cause But Flaccid Muscular Paralysis

H. Böhnel[1] and F. Gessler[2]

[1] Institute for Tropical Animal Health, Georg-August-University
Kellnerweg 6, 37077 Göttingen, Germany
e-mail hboehne@gwdg.de

[2] Institute for Applied Biotechnology in the Tropics at the Georg-August-University
Kellnerweg 6, 37077 Göttingen, Germany
e-mail fgessle@gwdg.de

The cause of botulism is the action of botulinum neurotoxins. The classical form is an intoxication, and since 1976 intestinal toxico-infections are known as well (shaker foal syndrom; infant botulism).

The influence on the endocrinium was neglected although visible affections of endocrinal glands (e.g., salivary glands) were reported.

Since 1922 BoNT was suspected of causing equine grass sickness, a combination of digestive and neural disorders, normally with lethal outcome. A significant change in the coeliacal ganglia indicates a connection of the autonomous nervous system and intestinal contents.

In 2001 a new clinical form of a chronic disease in cattle was described as visceral botulism. This is a herd problem leading to reduced milk production, different disorders e.g., emaciation, laminitis, mastitis, abomasal displacement and eventually followed by death or euthanasia, and increased calf mortality. We tried to explain it as an affection of different neuronal networks, cholinergic nerves, the autonomous nervous system, and intestinal muscles.

Pharmacological research in exocytosis uses BoNT als means of impact. Reports show that BoNT may influence directly cells e.g., in the pankreas, or indirectly in other culture cells after permeabilisation.

Neuroimmunoendocrinological and psychoneuroimmunological connections exist between the continuous presentation of antigens to the immune system, the formation of antibodies and cytokines, like Il-1, and remote actions in different target systems as phagocytes, neurons of the CNS and others, and regulatory loops like the Hypothalamo-Pituitary-Adrenal axis.

An unbiased approach should lead to a global understanding of the effect of BoNT in animal and human organism.

Hn-33 Enhances the Endopeptidase Activity of Botulinum Neurotoxin A and E Against Brain Synaptosomal SNAP-25

Shashi Kan Sharma[1] and B. R. Singh[2]

[1] U.S. Food and Drug Administration, Center for Food Safety and Applied Nutrition
College Park, MD 20740

[2] Department of Chemistry and Biochemistry, and Center for Marine Science and Technology
University of Massachusetts Dartmouth
Dartmouth, MA 02747

In botulism disease, neurotransmitter release is blocked by a group of structurally related neurotoxin proteins produced by *Clostridium botulinum*. Botulinum neurotoxins, (BoNT, A-G), enter nerve terminals and irreversibly inhibit exocytosis via their endopeptidase activities against synaptic proteins SNAP-25, VAMP and Syntaxin. Type A *C. botulinum* secretes the neurotoxin along with 7 other proteins called Neurotoxin Associated Proteins (NAPs). Here we report that Hn-33, one of the NAP components enhances the endopeptidase activity of BoNT/A and BoNT/E *in vitro* and *in vivo*. BoNT/A appears to have a stronger endopeptidase activity. Within 30 min, reduced BoNT/A and BoNT/E cleaved SNAP-25 by 22 % and 10 %, respectively. Addition of Hn-33 separately to even non-reduced BoNT/A and BoNT/E enhanced their *in vitro* endopeptidase activity by 21.5 and 25-folds, respectively. Rat brain synaptosome expressing SNAP-25 was used for *in vivo* study. In rat brain synaptosomes, reduced BoNT/A and BoNT/E cleaved synaptosomal SNAP-25 protein by 20 % and 15 %, respectively. Addition of Hn-33 separately to non-reduced BoNT/A and BoNT/E enhanced their endopeptidase activities by 13 and 15-fold, respectively. We believe that Hn-33 could be used as an activator of the neurotoxin, which is currently used as a therapeutic agent against several neuromuscular disorders.

Persistence of Botulinum Neurotoxin Serotypes A and E in Rat Extensor Digitorum Longus Muscle

Michael Adler[1], Sharad S. Deshpande[2], Robert E. Sheridan[1], David Mann[1] and James P. Apland[1]

[1] Neurotoxicology Branch, Pharmacology Division,
US Army Medical Research Institute of Chemical Defense
Aberdeen Proving Ground, MD 21010

[2] Battelle Memorial Institute, Edgewood Operations
Bel Air, MD 21015

Botulinum neurotoxin serotypes A (BoNT/A) and E (BoNT/E) inhibit neurotransmitter release from peripheral cholinergic nerve terminals by cleaving different sites on SNAP-25, a protein involved in synaptic vesicle docking and exocytosis. Since recovery from BoNT/A is protracted, but reversal of BoNT/E intoxication is relatively rapid, it was of interest to determine whether the duration of intoxication reflects persistent toxin activity or persistence of truncated SNAP-25 (aa 1-197) resulting from the action of BoNT/A. In a previous study, we concluded that persistence reflected enzymatic activity of the light chain. However, since this study was performed with toxin complex and used equipotent doses of BoNT/A and /E, it was necessary to determine if the results applied to pure toxin and conditions of relatively low BoNT/A and high BoNT/E. Extensor digitorum longus (EDL) muscles from rats were injected locally with 1.25 units of BoNT/A, 40 units of BoNT/E or 1.25 units of BoNT/A followed by 40 units of BoNT/E 48 hr later. Muscle tensions were elicited *in situ* in response to supramaximal stimulation of the peroneal nerve to monitor recovery from BoNT intoxication. Tensions returned to 16.6 and 92.1% of control, respectively, 4 and 14 days after injection of BoNT/E. In contrast, tensions in muscles injected with BoNT/A returned to only 4.4 and 20.8% of control at these time points. The time course for recovery was comparable to our previous study. Preparations injected sequentially with BoNT/A followed by BoNT/E exhibited slow recovery times resembling those recorded in the presence of BoNT/A alone. If the slower recovery from BoNT/A intoxication were determined by the longer persistence of the truncated SNAP-25 resulting from BoNT/A action, injection of a relatively high dose of BoNT/E should have converted BoNT/A-truncated SNAP-25 to the species appropriate for BoNT/E action (aa 1-180) and thus accelerated recovery. Since the sequential exposure experiments indicate that recovery from BoNT intoxication was not shortened by exposure to serotype E, the duration of BoNT/A intoxication appears to be determined by the intracellular stability of catalytically active BoNT/A light chain rather than by difficulty in removing BoNT/A-truncated SNAP-25 from the nerve terminal.

Molecular Steps in Botulinum Action — Known Knowns and Known Unknowns

Bal Ram Singh

Department of Chemistry and Biochemistry
University of Massachusetts Dartmouth
Dartmouth, MA 02747

The three major fields of botulinum toxin impact are food poisoning, bioweapons, and therapeutic applications. While there is a common biochemical mechanism involved in its action, routes of entry and possible molecular forms of the toxin could vary under the three impact conditions. The four-step molecular mechanism involved in botulism or botherapy (therapy with botulinum) are only partially understood. Relative importance of each of the steps in the biological effect of botulinum is critical for designing preventive and therapeutic measures against botulism or to prepare efficacious therapeutic product from botulinum agents. For example, neurotoxin associated proteins (NAPs) are known to protect the toxin from adversarial environmental conditions and increase its stability, and are also known to assist in the entry of the toxin across mucosal layer of the intestinal tract. NAPs also act as effective formulating agents for botulinum-based therapeutic reagents. How critical then NAPs are for botulism and botherapy? Similar, how critical is the presence of a specific protein receptor at the nerve terminals? Available data on these and other steps involved in the biochemical manifestation of the botulinum toxin will be examined in this presentation.

Supported by the U.S. Army Medical Research and Material Command under Contract No. DAMD17-02-C-001.

Inhibition of Toxin Production of Nonproteolytic *Clostridium botulinum* Type B in Cooked Sausages by Nitrite

R. Keto-Timonen, M. Lindström, M. Nevas, and H. Korkeala

Department of Food and Environmental Hygiene,
Faculty of Veterinary Medicine, University of Helsinki
Helsinki, Finland
riikka.keto@helsinki.fi

The use of nitrite in meat curing has become a controversial issue since nitrite can serve as a precursor of carcinogenic nitrosamines. The effect of three different levels of nitrite (0, 75 and 120 mg/kg) on the growth and toxin production of nonproteolytic *Clostridium botulinum* type B in cooked sausages (diameter 75 mm) was studied. The spore inoculation consisting of equal numbers of three nonproteolytic type B strains (Eklund 2B, Eklund 17B, and Hatheway 706 B) was adjusted at a level of log 2.0 CFU/g of raw sausage emulsion. The core temperature of the sausages reached 72°C during the heat treatment. The sausages were sliced and vacuum-packaged immediately after cooking and stored at 8°C for 5 weeks. Five samples per each nitrite level were analysed for the presence of *C. botulinum* type B 1, 3, and 5 weeks after manufacturing by PCR. The quantification was based on a five-tube most probable number series. The presence of botulinum neurotoxin was analysed by a mouse bioassay. The mean botulinal count in sausages produced without nitrite increased during the storage time (Table 1). In addition, all sausages produced without nitrite were toxic on week 3 and 5. Toxigenesis was not observed in sausages produced with 75 or 120 mg/kg nitrite by the end of the storage period. The results of the present study indicate that 75 mg/kg nitrite is sufficient to inhibit toxigenesis of nonproteolytic *C. botulinum* type B in cooked sausages.

TABLE 1. Cell count and toxigenesis of nonproteolytic *C. botulinum* type B in sausages with three levels of nitrite.

	Nitrite level (mg/kg)					
	0		75		120	
Storage time (weeks)	Cell count[a]	Toxi-genesis[b]	Cell count	Toxi-genesis	Cell count	Toxi-genesis
1	2.1 (1.3-2.8)	ND	2.5 (2.0-2.8)	ND	2.3 (1.9-2.7)	ND
3	2.1 (1.5-2.7)	5/5	2.1 (0.5-4.2)	0/5	1.9 (1.5-2.4)	0/5
5	2.8 (2.1-3.7)	5/5	1.6 (1.2-2.0)	0/5	2.0 (1.4-2.5)	0/5

ND = not determined
[a] Mean log MPN CFU/g estimate of cell count (CFU/g); minimum and maximum values for cell count in the parentheses
[b] Number of toxic samples/number of all samples analyzed

Formulating Low-Acid Foods for Botulinal Safety

Ann Larson, Kathleen Glass, Lindsey McDonnell, and Eric Johnson

Food Research Institute
University of Wisconsin-Madison
Madison, WI 53706

Formulating foods to consider intrinsic and extrinsic factors is critical for controlling *C. botulinum* growth and toxin production in minimally processed low acid foods. Well-known examples of formulation safe foods include shelf-stable cured luncheon meat products and many pasteurized process cheese spreads and sauces. More recently, certain modified atmosphere packaged baked goods and cream cheese-based spreads have appeared on the market that ensure safety using combinations of mild heat and strict control of formulation. Similarly, refrigerated, processed foods processed to reduce competitive microflora should use combinations of water activity, pH, and antimicrobials to inhibit growth of psychrotrophic strains of *C. botulinum* or to ensure safety in the event of temperature abuse. Antimicrobials with potential uses against *Clostridium botulinum* are bacteriocins, organic acids and their salts, lysozyme, nitrite, phosphates, and sorbates. This presentation will discuss specific combinations of hurdles that inhibit botulinal growth and toxin production in shelf-stable and refrigerated foods.

Thermal Inactivation of Nonproteolytic *Clostridium botulinum* Type E in Fish

Miia Lindström[1], Mari Nevas[1], Sebastian Hielm[1],
Michael W. Peck[2], and Hannu Korkeala[1]

[1]Department of Food and Environmental Hygiene, Faculty of Veterinary Medicine,
University of Helsinki, Helsinki, Finland

[2]Institute of Food Research, Norwich Research Park
Colney, Norwich, UK

Vacuum-packaged hot-smoked fish products have frequently been traced as vehicles for type E human botulism outbreaks. The Northern waters are heavily contaminated with nonproteolytic *Clostridium botulinum* type E spores, and subsequently the fish caught in such areas contain spores. The heat treatments commonly employed in the hot-smoking industry are too low to eliminate nonproteolytic *C. botulinum* spores, and in a previous study 5% of vacuum-packaged hot-smoked fish products sold in Finland contained spores of *C. botulinum* type E. Vacuum-packaging allows extended shelf lives and provides *C. botulinum* with optimal conditions for growth and toxin production. NaCl concentrations of more than 3.5% (w/v) would inhibit the growth of nonproteolytic *C. botulinum*, but due to health aspects the NaCl content of many fish products sold in the Nordic countries are in the order of 1 to 2%. Moreover, nonproteolytic *C. botulinum* may grow at 3°C, whereas the true storage temperatures at the retail and consumer level may be up to 10°C. Thus, the control of growth and toxin production by nonproteolytic *C. botulinum* type E in fish products should preferably be established by enhanced heat processing.

In Europe, the current guidelines for the heat processing of refrigerated processed foods of extended durability, including vacuum-packaged hot-smoked fish, recommend a 6D process to be used. Such a process would reduce the number of *C. botulinum* spores by a factor of 10^6. Time-temperature combinations of 52 min 85°C, 10 min 90°C, and 3 min 95°C have been proposed, but such heat treatments have since been shown to often require additional hurdle factors to ensure product safety. In addition, lysozyme has been shown to greatly increase the spore heat resistance, and its presence in a number of foods increases the risk of survival and subsequent growth and toxin production by nonproteolytic *C. botulinum*.

A high relative humidity (RH) has been reported to enhance the thermal destruction of nonproteolytic spores of *C. botulinum* type E. In this study, heat treatments of at least 42 min at 85°C combined with smoke house RH of 70% were shown to control the growth and toxin production of spores by a factor of 10^6 within 5 weeks in the presence of lysozyme at 8°C. On the contrary, the same heat treatment combined with 10% RH resulted in growth and toxin production from spores of nonproteolytic *C. botulinum* type E within 5 weeks at 8°C. In a sensory evaluation the safely prepared fish products were perceived to be cooked to the right degree. Thus, the use of heat processes of at least 42 min at 85°C combined with at least 70% RH should be strongly encouraged in the hot-smoking industry.

An Exposure Assessment of the Foodborne Botulism Hazard in Gnocchi, a Minimally Processed Refrigerated Potato Product of Italian Origin

P. K. Malakar, G. C. Barker, M. Del Torre, M. L. Stecchini, S. C. Stringer and M. W. Peck

Institute of Food Research, Norwich Research Park
Colney, Norwich, NR4 7UA, England, UK

Sales and consumption of refrigerated processed foods of extended durability (REPFEDs) have increased many fold in Europe over the last few years. These foods include chilled pasta and gnocchi (a REPFED of Italian origin). Non-proteolytic *Clostridium botulinum* is a major hazard for the safety of these ready-to-eat foods, because of its ability to survive a mild heat treatment and produce toxin under refrigeration conditions. This presentation will describe the use of the techniques of quantitative risk assessment to conduct an exposure assessment of the foodborne botulism hazard presented by non-proteolytic *C. botulinum* in gnocchi. This approach allows an overall evaluation of the safety of the product, and indicates that the product is very safe with regard to the foodborne botulism hazard presented by non-proteolytic *C. botulinum*.

Program: Thursday, October 24, 2002 Alumni Lounge, Pyle Center [concurrent with Social hour reception]

Session	Title	Presenter	Affiliation
Poster Session, 5:30 – 6:30 p.m.	Sensitivity of Dorsal Root Ganglion Neurons to Botulinum Neurotoxins: A Comparative Study	Keith A. Foster	CAMR, Salisbury, UK
	Genetic Characterization of *Clostridium botulinum* Type A Strains Isolated in Italy: Preliminary Results	Giovanna Franciosa	Istituto Superiore della Sanitá, Rome, Italy
	Identification of Type A, B, and E Botulinum Toxin Genes and of Neurotoxigenic Clostridia by Denaturing HPLC (*DHPLC*)	Giovanna Franciosa	Istituto Superiore della Sanitá, Rome, Italy
	QSAR of Buforin Analog Inhibitors of Botulinum Toxin B	Gregory E. Garcia	WRAIR, Silver Spring, MD
	Comparison of Methods to Evaluate the Botulinal Safety of Foods	Ann Larson and Eric Johnson	Food Research Institute, University of Wisconsin, Madison, WI
	A Bayesian Belief Representation for the Exposure of Consumers to Non-Proteolytic *Clostridium botulinum* in Gnocchi	Michael W. Peck	Institute of Food Research, Norwich, England
	Germination, Emergence and Time to First Division from Single Spores of Non-proteolytic *Clostridium botulinum* Strain 17B	Michael W. Peck	Institute of Food Research, Norwich, England
	Phospholipase A2 and Rho B as Pharmacological Antagonists of Botulinum Toxin A Poisoning	Prabhati Ray	Walter Reed Army Institute of Research, Silver Spring, MD
	Recombinant Botulinum Neurotoxin Type A Vaccine Protects Against Disparate Type A Strains	Theresa J. Smith	USAMRIID, Fort Detrick, MD

Sensitivity of Dorsal Root Ganglion Neurons to Botulinum Neurotoxins: A Comparative Study

K. A. Foster, J. A. Chaddock, E. R. Kirby, H. J. Moulsdale

Centre for Applied Microbiology and Research
Porton Down, Salisbury, Wiltshire, SP4 0JG, UK
keith.foster@camr.org.uk

We have previously reported the differential sensitivity of embryonic rat dorsal root ganglia neurons to different serotypes of botulinum neurotoxins (Welch et al, 2000, Toxicon, 38, 245-258). Now we have extended these studies to characterise these differences more fully. In addition to assessing relative potencies in terms of both substrate cleavage and inhibition of multiple neurotransmitter types, we have also investigated the duration of action of the different serotypes. The results indicate that there are significant differences in the effects of the neurotoxin serotypes in different neuronal populations. The relationship between serotype, mode of internalisation, and duration of activity is discussed

Identification of Type A, B, and E Botulinum Toxin Genes and of Neurotoxigenic Clostridia by Denaturing HPLC (*DHPLC*)

Giovanna Franciosa[1], Manoocheher Pourshaban[1],
Alessandro De Luca[2], Bruno Dallapiccola[2], and Paolo Aureli[1]

[1]National Reference Center for Botulism, Food Laboratory,
Istituto Superiore della Sanità, Rome, Italy

[2]Istituto CSS-Mendel, Rome, Italy

Denaturing high-performance liquid chromatography (*DHPLC*) is a recently developed technique capable of detecting nucleotide differences in a PCR product in comparison with a "wild-type": sequence variations are revealed as different peak profiles.

It has recently proven successful for identifying bacteria, specifically those with a high potential to be used as agents in bioterrorism.

We applied here this technique for identification of botulinum neurotoxin gene type. Degenerate primers were used to amplify a 260bp fragment from the type A, B and E botulinum toxin genes from *Clostridium botulinum* and neurotoxigenic *C. butyricum* type E; PCR amplicons were subjected to both DHPLC analysis and sequencing. Unique DHPLC peak profiles were obtained with each different type of botulinum toxin gene fragment, which related well with nucleotide differences in the sequences.

We then evaluated the capability of this technique to identify the neurotoxigenic organisms at the genus and species level. To this end, a specific short region of the 16S rDNA which shows genus-specific and in some cases species-specific heterogeneity was amplified from different food-borne pathogens (*C. botulinum*, *C. butyricum*, *Listeria monocytogenes*, *Enterococcus fecalis*, *Salmonella enteritidis*, *Bacillus cereus*) and subjected to DHPLC analysis. Differences in the peak profiles were observed with each genus and species, thus demonstrating that the technique could be a reliable alternative to sequencing for fast identification of foodborne pathogens, and specifically of neurotoxigenic clostridia.

Genetic Characterization of *Clostridium botulinum* Type A Strains Isolated in Italy: Preliminary Results

Giovanna Franciosa, Francesca Floridi, Lucia Fenicia,
Manoocheher Pourshaban, Fabrizio Anniballi, and Paolo Aureli

National Reference Center for Botulism
Food Laboratory
Istituto Superiore della Sanità, Rome, Italy

During the last 10 years, *Clostridium botulinum* type A has been isolated from 18 cases of human botulism in Italy (14 foodborne botulism, 3 infant botulism, and 1 infant-like botulism in an adult): 3 of them, all foodborne, were fatal. Moreover, a recent survey for contamination of dairy products with *C. botulinum* spores and toxin demonstrated that *C. botu*linum type A is the most frequent spoiler of this kind of products.

We analyzed here by PCR some representative *C. botulinum* type A strains from our collection for presence of BoNT/A and BoNT/B genes, and of HA-35 and p47 genes: the latter genes are components of the cluster of genes encoding proteins of the botulinum toxin progenitor complexes. A total of 40 strains of *C. botulinum* type A, 18 of which were from clinical specimens, 18 randomly selected among those isolated from dairy products of different brands and production lots, 1 from a honey sample from the market, and 1 from a sea salt sample, were included in this study; strains 62A (type A) and 3517 [type A(B)] from the CDC collection were also included, as controls. All strains harbored the type A toxin gene, as expected; however, 4 of them (3 strains from distinct cases of foodborne botulism, one of which fatal, and 1 from a sea salt sample) also possessed the type B toxin gene. A double-step PCR specifically designed to detect one of the mutations (a 6 nucleotide deletion) that inactivates the type B toxin gene in *C. botulinum* type A(B) strains demonstrated that the gene from the 4 strains was not mutated, and actual production of minor amounts of type B toxin by these strains was confirmed by mouse bioassay. Hence, 4 of the 38 strains were re-classified as *C. botulinum* sub-type Ab. The 4 strains of *C. botulinum* subtype Ab were positive for both HA35 and p47 genes, consistently with findings on the organization of the genetic clusters of the BoNT/A and BoNT/B progenitor complexes from *C. botulinum* type Ab and A(B) strains. PFGE analysis of these 4 strains by 2 enzymes showed 3 different profiles with each enzyme, one of which shared by two strains (one from the sea salt sample and one from a clinical specimen).

The remaining 34 strains of *C. botulinum* type A from our collection lacked the HA35 gene, while they were positive for the p47 gene: hence, all *C. botulinum* type A included in this study belong to the "type A2", as strain Kyoto F isolated in Japan.

QSAR of Buforin Analog Inhibitors of Botulinum Toxin B

Gregory E. Garcia, Deborah R. Moorad, and Richard K. Gordon

Walter Reed Army Institute of Research, Division of Biochemistry
Silver Spring, MD 20910

Buforin I (B-I) is an antimicrobial peptide that contains 39 amino acids, and was first isolated from stomachs of Asian toads. B-I contains a VAMP2 amino acid QF cleavage site for the endopeptidase activity of BotNT/B, but shares only 18% primary sequence with VAMP2, which is the natural substrate cleaved by BoNT/B. We previously reported that B-I did not act as a substrate for BoNT/B, but B-I dose-dependently and competitively inhibited BoNT/B activity, yielding an IC_{50} of 1 μM. To further define the minimum requirements for this class of peptide inhibitor, we examined analogs of substance P, an 11 amino acid peptide with the QF bond but much weaker inhibition of BotNT/B. The peptide analogs were generally non- or weak inhibitors, as were chimeric peptides composed of substance P upstream and the B-I sequence downstream of the QF site until almost the complete C-terminal amino acid sequence of B-I was added. This peptide (05P) was 24 amino acids shorter than B-I and yet retained significant inhibition, indicating that rational design improvements can be effective. A CYS B-II-SubP chimera peptide exhibited greater inhibition of BoNT/B than a LYS analog, indicating that the CYS moiety may interact with Zn^{2+} at the active site of the endopeptidase. Buforin pharmacokinetics in mice indicated that upon IM thigh injection, the level of the radiolabelled B-I remained above 70% level even after 3 hrs. In conclusion, QSAR studies of B-1 analogs suggest that B-I has potential as an inhibitor of and prophylactic treatment for BoNT/B intoxication.

Comparison of Methods to Evaluate the Botulinal Safety of Foods

Ann Larson and Dr. Eric Johnson

Food Research Institute, University of Wisconsin–Madison
Madison, WI 53706

In traditional botulinal challenge studies to evaluate the ability of *Clostridium botulinum* to grow and produce toxin in foods, inoculated foods are periodically assayed by mouse bioassay during incubation to detect the presence of botulinum neurotoxin. The overall objective of this research was to examine the reliability, sensitivity, and accuracy of alternate testing methods to determine the botulinal safety of foods. These methods included monitoring growth of inoculated *C. botulinum* (or nonpathogenic *Clostridium sporogenes* as a surrogate) using plate counts, monitoring for visible gas production in samples, or testing for botulinum toxin in samples using an enzyme-linked immunosorbent assay (ELISA) method. These alternate methods were compared in parallel to the mouse bioassay.

Challenge studies were conducted in 10 systems (nine foods and TPGY broth medium) inoculated with spores of either *C. sporogenes* PA3679 or a mixture of proteolytic *C. botulinum* strains. Foods were incubated at 18-27°C and tested periodically for numbers of *C. botulinum* or *C. sporogenes* using differential agar media, visible gas production, and for the presence of botulinum toxin using both an ELISA method and the mouse bioassay. Additionally, the same foods and TPGY broth were inoculated with a mixture of nonproteolytic *C. botulinum* spores, incubated at 12-15°C, and samples monitored during incubation for toxin production, visible gas production, and numbers of *C. botulinum* as described above.

Botulinum toxin was detected by mouse bioassay during incubation in all media and food systems inoculated with proteolytic *C. botulinum*. Significant increases in proteolytic *C. botulinum* plate counts were detected during incubation in six of ten systems tested. Significant increases in *C. sporogenes* plate counts were detected during incubation in seven of ten systems tested. Significant increases in nonproteolytic *C. botulinum* plate counts were detected in only two of six food or media systems supporting toxin production under refrigeration or mild abuse conditions. The ELISA method used detected botulinum toxin in 123 of 128 toxic (by mouse bioassay) samples tested, with false positive results for two of 20 nontoxic samples tested.

A Bayesian Belief Network Representation for the Exposure of Consumers to Non-Proteolytic *Clostridium botulinum* in Gnocchi

P. K. Malakar, G. C. Barker, S. C. Stringer, M. Del Torre,
M. L. Stecchini & M. W. Peck

Institute of Food Research, Norwich Research Park, Colney
Norwich, NR4 7UA, England, UK

In Europe, sales of refrigerated processed foods of extended durability (REPFEDs) have increased many fold over the last few years. Examples of these ready-to-eat foods include chilled pasta and gnocchi. Non-proteolytic *Clostridium botulinum* is identified as a major hazard for the safety of these foods, because of its ability to survive a mild heat treatment and produce neurotoxin under refrigeration conditions. This presentation will describe a Bayesian Belief Network representation for the exposure of consumers to non-proteolytic *C. botulinum* in gnocchi, a minimally processed potato product of Italian origin. A combination of data from various sources has shown that materials quality, thermal processing and two growth hurdles (low temperature storage and sorbic acid) combine to make this product very safe with regard to the foodborne botulism hazard presented by non-proteolytic *C. botulinum*.

Germination, Emergence and Time to First Division from Single Spores of Non-Proteolytic *Clostridium botulinum* Strain 17B

M. D. Webb, S. C. Stringer, R. B. Piggott,
J. Baranyi and M. W. Peck

Institute of Food Research, Norwich Research Park, Colney
Norwich, NR4 7UA, England, UK

If given an opportunity to grow, *Clostridium botulinum* can produce an extremely potent neurotoxin, ingestion of which causes severe illness or death. It is particularly important in heated products as competing vegetative flora is eliminated. In such products growth is likely to initiate from just a few spores. Traditionally, spore germination has been investigated in populations, but this gives no indication of the heterogeneity of time to germination, emergence or first division.

The aim of this work was to quantify time to germination, emergence and first cell division in individual spores of *C. botulinum* so that rate-limiting steps during spore germination could be identified and biovariability determined. Events during germination and outgrowth of individual spores of non-proteolytic *C. botulinum* strain Eklund 17B were recorded over time with phase-contrast microscopy coupled to automated image analysis.

When spores were germinated at room temperature in PYGS agar the percentage germination (measured as the change from phase bright to phase dark) was high (90%). There was great variation in the time to germination, from 20 min to 450 min, although most spores germinated within 100 min. Vegetative cells emerged from germinated spores explosively so were easily detected using the image analysis system. Biovariability was again evident in the time to emergence (150 min to 500 min), but there was only a weak correlation with the time to germination. First cell division (based on cell length measurement) occurred between 190 min and 550 min. Again any correlation between times to cell division and germination or emergence was weak.

The data presented here demonstrates that there is considerable heterogeneity within this spore population with respect to the distribution of germination and outgrowth events. These events (germination, emergence and first cell division) contribute to lag phase, so it is important to quantify this biovariability if predictions of growth from spores in food is to be improved.

Phospholipase A_2 and Rho B as Pharmacological Antagonists of Botulinum Toxin A Poisoning

Prabhati Ray, Hiroshi Ishida and Xieping Zhang

Walter Reed Army Institute of Research
Silver Spring, MD 20910
Prabhati.ray@na.amedd.army.mil

Botulinum toxin type A (BoNT/A) is one of the most serious biological threats faced by the U. S. military and allied forces. The challenges presented by the complexity of BoNT/A action demand that we explore new approaches in our efforts to develop strategies for effective prophylaxis and therapy of BoNT/A poisoning. BoNT/A inhibits Ca^{2+}-dependent acetylcholine (ACh) release (neuroexocytosis) at peripheral neuromuscular junctions and causes fatal paralysis. To explain the mechanism of action of BoNT/A, previously we reported that (a) in nerve growth factor (NGF)-differentiated PC12 cells, arachidonic acid (AA) release is associated with ACh release, (b) BoNT/A inhibits both release processes, and (c) AA itself or a phospholipase A_2 (PLA_2) activator can cause ACh release in BoNT/A poisoned cells in which SNAP-25 has supposedly been hydrolyzed. These results suggest that (a) besides the vesicle fusion proteins such as SNAP-25, another phospholipase-dependent mechanism regulates exocytosis, and (b) when the SNAP-25 mediated pathway is inhibited e.g., due to BoNT/A, it is possible to induce exocytosis via the PLA_2 mediated pathway. As an additional support for this PLA_2 mediated mechanism, here we report the results of our investigation on the effect of PLA_2 over-expression on inhibition of ACh exocytosis due to BoNT/A light chain (LC) in PC12 cells. BoNT/A LC cDNA was cloned into a plasmid encoding the green fluorescence protein (GFP). PC12 cells were transfected with this construct to study BoNT/A LC expression and its effect on SNAP-25 (hydrolysis). Successful transfection and expression of BoNT/A LC were validated by (a) a fluorescence microscopic (red:SNAP-25-rhodamine and green:LC-GFP) assay of SNAP-25 hydrolysis, and (b) an inhibition of K^+-stimulated ACh release in transfected cells. Over-expression of PLA_2 alone in these BoNT/A LC transfected cells augmented the stimulated release of ACh and AA. PLA_2 over-expression also effectively prevented the inhibition of stimulated ACh and AA release due to BoNT/A LC.

Phospholipases act on membrane phospholipids to generate AA and lysophosphatidic acid (LPA). LPA activates Rho GTPases, which have been implicated in mechanisms correlating actin cytoskeletal organization and exocytosis. In NGF-differentiated PC12 cells, over-expression of wild type RhoB alone caused actin reorganization and enhanced neuroexocytosis. BoNT/A inhibited LPA-induced actin reorganization and, therefore, ACh exocytosis. RhoB over-expression prevented the BoNT/A inhibitory effect on actin reorganization, and maintained LPA stimulated ACh exocytosis. Over-expression of dominant negative type RhoB inhibited LPA stimulated neuroexocytosis, and was ineffective to counteract BoNT/A inhibition of stimulated ACh exocytosis. These results suggest that PLA_2 and RhoB may serve as possible pharmacological antagonists of BoNT/A poisoning.

Recombinant Botulinum Neurotoxin Type A Vaccine Protects Against Disparate Type A Strains

Theresa J. Smith[1], James D. Marks[2],
Vicki A. Montgomery[1], and Leonard A. Smith[1]

[1]Toxinology and Aerobiology Division,
U. S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

[2]Department of Anesthesiology, University of California,
San Francisco General Hospital, San Francisco, CA[2]

Recombinant botulinum neurotoxin vaccines comprising the binding domain of the heavy chain (rBoNT(Hc)) have been shown to be highly protective against challenge with the botulinum neurotoxins (BoNT). We previously characterized the immune response to a rBoNT(Hc) vaccine constructed from the Hall type A strain and reported its protective abilities in mice challenged with type A Hall toxin. Since type A strains can differ significantly at the amino acid level, we compared the immune response and protective capacity of the Hall rBoNT/A(Hc) vaccine against challenge with a BoNT/A isolate from the Wisconsin Food Research Institute (BoNT/A FRI-honey) which differs from the Hall strain by ~10% at the amino acid level. Despite the significant sequence difference, high titer antibody was generated which cross reacted with BoNT/A FRI-honey resulting in complete protection of mice challenged with up to 100,000 mouse LD_{50}s of BoNT/A FRI-honey. We conclude that a rBoNT/A(Hc) vaccine is capable of providing broad protection against genetically diverse BoNT/A toxins.

Program: Friday, October 25, 2002 Auditorium, Pyle Center

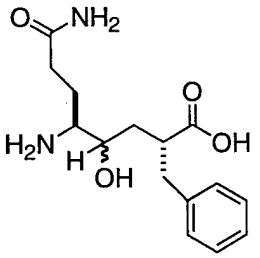
Session	Title	Presenter	Affiliation
Clinical Steve Arnon 8:00 – 9:30 a.m.	Design and Synthesis of Inhibitors of Botulinum Toxin Metalloproteases	Daniel Rich	Pharmacy, UW-Madison
	Antinociceptive Mechanism of BOTOX	Roger Aoki	Allergan, Irvine, CA
	Botulinum Toxin 2002: Clinical Update	Mitchell Brin	Allergan, Irvine, CA
- - - - - - Break 9:30 – 10:00 a.m. - - - - - -			
Hot Topics Eric Johnson 10:00 – 11:00 a.m.	Sequencing the Genome of Proteolytic *Clostridium botulinum* ATCC 3502 (Hall A)	Marjon Bennik	Institute of Food Research, Norwich, England
	Botulinum Toxin — Promotion to "Respectability"	Susan Maslanka	CDC, Atlanta, GA
Wrap-up session, Eric Johnson 11:00 a.m. – 12:00 p.m.			
- - - - - - Adjourn at noon - - - - - -			

Design and Synthesis of Inhibitors of Botulinum Toxin Metalloproteases

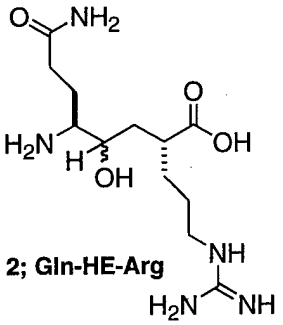
Daniel H. Rich[ab], Matthias Brewer[a], Chanokporn Sukonpan[b], Thorsten Oost[ab], Michael Goodnough[c], William Tepp[c] and Eric A. Johnson[c]

[a]Department of Chemistry, University of Wisconsin–Madison
[b]School of Pharmacy, University of Wisconsin–Madison
[c]Food Research Institute, University of Wisconsin–Madison

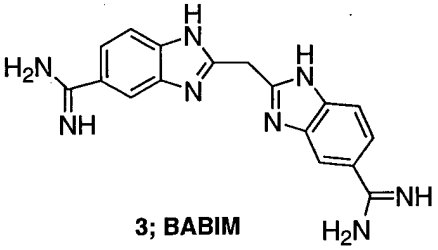
Botulinum Toxin (BoNT) Metalloproteases and related proteases are the most selective proteases known. In order to understand the molecular basis for the extraordinary selectivity and to develop non-peptide inhibitors that might be useful against BoNT toxicity, we have designed and synthesized two distinct classes of inhibitors. X-ray crystal structures by others suggest that the native enzymes exist in a catalytically **incompetent** form that must be activated by substrate binding. In order to characterize the postulated substrate-induced conformational changes, we synthesized a series of transition-sate analog inhibitors (TSI) in which the dipeptide cleavage site has been replaced by isosteric tetrahedral intermediate analogs within the minimal substrate peptide sequence [35-mer and 17-mer for BoNT/B and /A respectively]. The synthesis of hydroxyethylene analogs of –Gln-Phe- (**1**) and –Gln-Arg- (**2**) suitable for use in solid phase synthesis will be shown to illustrate the strategy. The synthesis, characterization and inhibition kinetics for five series of compounds against holotoxins BoNT/B and /A will be reported. In addition, our discovery of the inhibition of BoNT/B metallo protease by BABIM (**3**) will be described. Detailed kinetics of inhibition of BoNT/B holotoxin will be reported. The effect of zinc ion concentration on enzyme kinetics and inhibitor potency will be described. The potential application of BABIM-derived inhibitors against anthrax lethal factor will be discussed.



1; Gln-HE-Phe



2; Gln-HE-Arg



3; BABIM

Antinociceptive Mechanism of BOTOX

Roger Aoki

Allergan Inc
Irvine, CA 92612

Aim of Investigation: To investigate the mechanism of antinociceptive effect of subcutaneous BOTOX® in the rat formalin model by assessing its effect on formalin-induced local glutamate release, electrophysiological activities of dorsal horn neurons and the expression of fos-like immunoreactivity in the spinal cord of rats.

Methods:
BOTOX® was subcutaneously administered into the rat paw 1 day before 5% 50 µl s.c. formalin challenge. Extracellular recording of dorsal horn neurons was then performed. In the another group of BOTOX® treated rats, the formalin-induced glutamate release in the paw was collected and then measured by Mass Spectrometry. The effect of BOTOX® on formalin evoked c-fos expression was assessed 2 hours after formalin injection.

Results:
Subcutaneous formalin (5%, 50 µl) produced a prolonged distinct biphasic response of dorsal horn convergent neurons. Pretreatment of rats with s.c BOTOX® significantly inhibited the formalin-induced electrophysiological activities of dorsal horn neurons in phase II but not phase I. Subcutaneous BOTOX® also dose dependently inhibited formalin-induced glutamate release in the paw and the expression of c-fos in the spinal cord.

Conclusion: These results demonstrate that the inhibition of neurotransmitter release from primary sensory neurons by s.c. BOTOX® mediates its antinociceptive effect. Local application of BOTOX® directly inhibits the peripheral sensitization produced by local neurotransmitter release, which then results in an indirect reduction in the central sensitization. Inhibition of nociceptive processing at peripheral site and at the spinal cord level may underline the mechanism of its application in treating migraine.

Botulinum Toxin 2002: Clinical Update

Mitchell Brin

Development and Therapeutic Area, BOTOX® & Neurology, Allergan, Inc.
and Neurology, University of California, Irvine
Irvine, CA

During the past 12 months, there have been over 200 publications on the clinical use of the various botulinum toxin serotypes, in addition to an international meeting. This brief clinical update will rely on the literature of recently reported clinical uses of the various botulinum toxin serotypes in the management of skeletal muscle (e.g., spasticity), glandular (e.g., hyperhidrosis), smooth muscle (e.g., bladder) and pain (e.g., headache) disorders, novel cosmetic applications and antigenicity.

Sequencing the Genome of Proteolytic *Clostridium botulinum* ATCC 3502 (Hall A)

M. H. J. Bennik[1], N. J. Minton[2], M. Elmore[2], B. Barrell[3],
R. Hutson[4], J. Parkhill[3] & M. W. Peck[1]

[1] Institute of Food Research, Norwich Research Park, Colney, Norwich, NR4 7UA, UK
[2] CAMR, Porton Down, Salisbury, Wiltshire, SP4 0JG, UK
[3] Pathogen Sequencing Centre, The Wellcome Trust Genome Campus, Hinxton, Cambridge, UK
[4] Department of Food Science, University of Reading, Reading, RG6 6BZ, UK

Funding from the Beowulf Genomics has enabled sequencing of the genome of proteolytic (Group I) *Clostridium botulinum* strain ATCC 3502 (Hall A) to be carried out at the Pathogen Sequencing Centre of the Wellcome Trust Genome Campus in UK.

The analysis is now at an advanced stage. At July 2002, there are **63,114 reads** totalling **32.349 Mb** and giving a theoretical coverage of **99.97%** of the genome. Finishing is in progress, and a database containing the latest assembly is available (http://www.sanger.ac.uk/Projects/C_botulinum/). This includes **16 contigs** > 1kb (**13 contigs** > 2kb) with a total size of **3.938 Mb**.

The presentation will summarise progress and highlight preliminary findings from the genome.

Botulinum Toxin — Promotion to "Respectability"
a.k.a.: Preparation for Response to Intentional Release of Botulinum Toxin

Susan Maslanka and Jeremy Sobel

Centers for Disease Control and Prevention
Atlanta, GA

Botulinum toxin has always received enormous respect from those who work with it. Those rare individuals who have devoted their life to studying the structure, function, physical characteristics, methods for detection of the toxin, or mechanisms to control its production in foods have marveled at the idiosyncrasies of this very potent toxin. However in spite of its potency, research support for this important agent has been limited. In the public's mind, botulism was a problem only for those who dared to prepare homecanned foods and not of great interest to people in general. Even therapeutic use of this toxin received little attention until it was found that it was the elixir of youth (or at least made us appear to be younger). Now also the public is becoming aware of what the "inner circle" always knew: Botulinum toxin is a potentially deadly weapon. Although still lagging behind other potential bioweapons, support for research programs directed toward better understanding the action of botulinum toxin, methods for sensitive rapid detection, and products for safe treatment of exposed individuals is beginning to increase. State health departments are receiving long needed financial aid to support the hiring and training of qualified personnel. Federal agencies are conducting test scenarios of an intentional release of botulinum toxin in order to identify gaps in our response capabilities. Additional CDC staff has been hired to support both our bioterrorism response capabilities, as well as our ability to respond to a natural event of botulism. An organized structure for response is being developed. And we are beginning to understand the need for better coordination among federal and state agencies. We are working toward more rapid detection methods that may aid in the establishment of additional test sites across the country. While currently adequate for most of our needs, new funds are being sought to increase our stockpile of antitoxin to be used for treatment of botulism cases. Federal funds are being made available to increase the research projects across the country. The increase in researchers is good in that it brings new ideas into the area; it is bad in that some of these individuals do not have a clear understanding of the hazards associated with working with botulinum toxin. With all the potential additional funds, there is a flurry of activity from individuals wanting to capture some of it. Thankfully, committees are being formed to help establish research priorities. While we hope a bioterrorism event using botulinum toxin will not occur, the ongoing preparations will be a tremendous help in our continued public health mission to respond to botulism outbeaks (natural or intentional).